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Nucleosome Assembly by a Complex of CAF-1 and Acetylated Histones H3/H4

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Summary

Chromatin assembly factor 1 (CAF-1) assembles nucleosomes in a replication-dependent manner. The small subunit of CAF-1 (p48) is a member of a highly conserved subfamily of WD-repeat proteins. There are at least two members of this subfamily in both human (p46 and p48) and yeast cells (Hat2p, a subunit of the B-type H4 acetyltransferase, and Msi1p). Human p48 can bind to histone H4 in the absence of CAF-1 p150 and p60. p48, also a known subunit of a histone deacetylase, copurifies with a chromatin assembly complex (CAC), which contains the three subunits of CAF-1 (p150, p60, p48) and H3 and H4, and promotes DNA replication-dependent chromatin assembly. CAC histone H4 exhibits a novel pattern of lysine acetylation that overlaps with, but is distinct from, that reported for newly synthesized H4 isolated from nascent chromatin. Our data suggest that CAC is a key intermediate of the de novo nucleosome assembly pathway and that the p48 subunit participates in other aspects of histone metabolism.

Introduction

In all eukaryotic cells, genomic DNA is packaged into nucleosomes. The nucleosome core consists of a highly conserved histone octamer (formed of two molecules each of core histones H2A, H2B, H3, and H4), around the surface of which ~145 bp of DNA is wrapped in 1.8 left-handed superhelical turns. In most species, native chromatin is further compacted into higher-order structures that are stabilized by the presence of a fifth type of histone molecule known as histone H1 (reviewed in Wolffe, 1995).

The process of chromatin assembly takes place during the S phase of the cell cycle where it is tightly coupled to the passage of the DNA replication fork (Lucchini and Sogo, 1995). During replication of eukaryotic chromosomes, parental histones are segregated onto the two nascent DNA duplexes in a seemingly random fashion (Sogo et al., 1986). The other half of the nucleosome complement is formed from newly synthesized histones in a process known as de novo assembly. De novo nucleosome assembly occurs in a stepwise manner: newly synthesized histones H3 and H4 are deposited first (Worcel et al., 1978; Smith and Stillman, 1991) and subsequently associate with either new or parental histone H2A.H2B dimers to generate mature histone octamers (Jackson, 1990). In contrast to parental nucleosome segregation which, at least in vitro, can be performed efficiently even in highly purified DNA replication systems (Ishimi et al., 1991), newly synthesized histones require additional cellular factor(s) for their regulated deposition into chromatin during DNA replication (reviewed by Kaufman, 1996).

Chromatin assembly factor 1 (CAF-1) is a protein originally purified from human cells on the basis of its ability to promote de novo nucleosome assembly during in vitro DNA replication of plasmids containing the simian virus 40 origin (Stillman, 1986; Smith and Stillman, 1989). CAF-1 purified from human cells is a complex of three polypeptides of apparent molecular masses 150, 60, and 50 kDa. cDNAs encoding the two large subunits of the CAF-1 protein have been isolated and shown to be essential for chromatin assembly in vitro (Kaufman et al., 1995).

Several lines of evidence argue that CAF-1 is a physiologically relevant chromatin assembly factor. First, the two large subunits of CAF-1 colocalize with sites of active DNA synthesis in human cell nuclei (Krude, 1995). Second, in contrast to other chromatin assembly factors, CAF-1 assembles nucleosomes preferentially onto replicating DNA molecules (Stillman, 1986). Finally, CAF-1 assembles newly synthesized histones H3 and H4 that are biochemically distinct from chromosomal histones (Smith and Stillman, 1991).

The discovery that newly synthesized histones H3 and H4 are modified by acetylation (Ruiz-Carrillo et al., 1975) and deacetylated shortly after their incorporation into chromatin (Jackson et al., 1976) has led to the proposal that transient acetylation of these histones may play a role in chromatin assembly. Supporting this notion, it was recently found that newly synthesized H4 isolated shortly after its deposition into chromatin exhibits a very specific pattern of lysine acetylation at residues 5 and 12 (Sobel et al., 1995). This pattern of acetylation is identical among three widely divergent species that have been examined (Drosophila, Tetrahymena, and human cells). This high degree of conservation suggests that acetylation of newly synthesized H4 may play some important function.

We describe herein the discovery and characterization of a chromatin assembly complex (CAC) that contains CAF-1 and posttranslationally modified histones H3 and H4. We also describe the identification of a cDNA clone for the small subunit of CAF-1 (CAF-1 p48); p48 is a member of a conserved subfamily of WD-repeat proteins. Although two nearly identical polypeptides are expressed in human 293 cells (p46 and p48), only p48 copurifies with CAC. Interestingly, p48 is also a subunit of human histone deacetylase HD1 (Taunton et al., 1996). In S. cerevisiae, a p48-related protein, termed Hat2p, is a subunit of a B-type histone H4 acetyltransferase (Parthun et al., 1996 [this issue of *Cell*]). The p48 family of proteins therefore appears to be involved in multiple aspects of histone function in widely divergent organisms.

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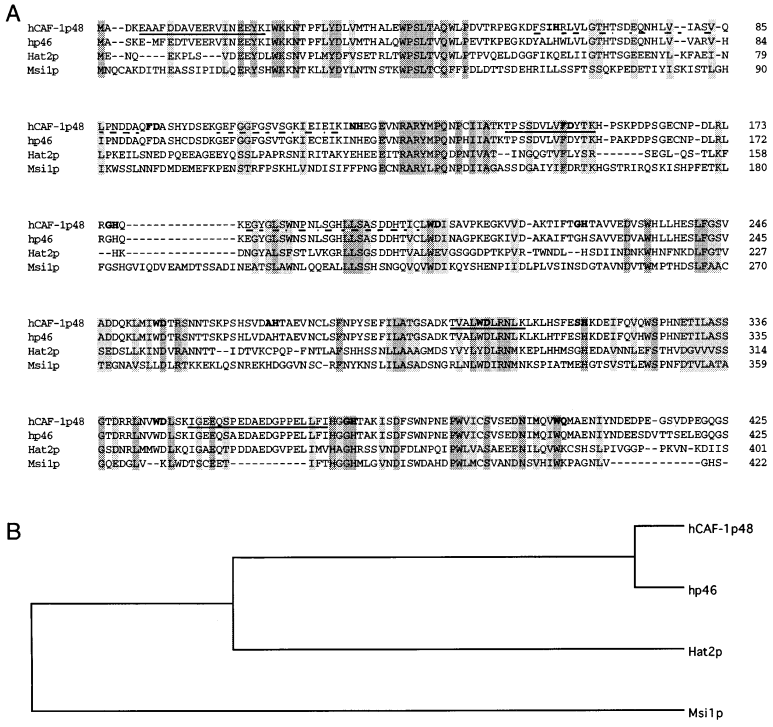

Figure 1. p46 and p48 Are Members of a Highly Conserved Subfamily of WD-Repeat Proteins

(A) Multiple sequence alignment of human p46 and p48, and S. cerevisiae Hat2p and Msi1p. The peptides of p48 obtained by amino acid sequencing of purified CAF-1 are underlined with a solid line, whereas the peptides derived from sequencing CAC p48 are underlined with a dashed line. Amino acids that are identical or similar in all four proteins are shaded dark or light, respectively. Amino acids characteristic of the seven WD-repeats are shown in bold in the p48 sequence. The distance between the H and F/W residue is 28 amino acids in each repeat of both p46 and p48.

(B) Phylogenetic tree showing that human p46 and p48 are more similar to S. cerevisiae Hat2p than to Msi1p.

Results

cDNA Cloning of the Small Subunit of CAF-1

To clone a cDNA for the small subunit of CAF-1, peptide sequences were obtained from human CAF-1 protein purified as described previously (Kaufman et al., 1995). These peptide sequences were used to design degenerate oligonucleotides for amplification of a cDNA fragment by reverse transcriptase–polymerase chain reaction (RT–PCR), and this probe was used to isolate a full-length cDNA clone from a HeLa cell library. Two distinct cDNA clones were isolated by this approach and they are known to encode retinoblastoma-associated protein RbAp46 and RbAp48 (Qian et al., 1993; Qian and Lee, 1995). As illustrated in Figure 1A, the p46 and p48 proteins are highly related to each other (89% identical). However, two of the four peptides sequenced from the 50 kDa subunit of CAF-1 are present in p48 and absent from the p46 protein (Figure 1A, peptides underlined with solid line). The cDNA for the p46 protein was isolated as a result of nucleotide sequence similarity between the p46 and p48 mRNAs (Qian et al., 1993; Qian and Lee, 1995).

Interestingly, p48 was identified recently as a polypeptide that is tightly associated with the catalytic subunit of human histone deacetylase HD1 (Taunton et al., 1996; see also Discussion, below). In addition, the human p46 and p48 proteins are very similar to budding yeast Hat2p (the histone recognition subunit of a B-type histone H4 acetyltransferase; Parthun et al., 1996) and, to a lesser extent, to yeast Msi1p (Figure 1B). Msi1p was originally identified as an antagonist of the Ras–cyclic AMP pathway in S. cerevisiae (Ruggieri et al., 1989), and it has been shown previously that both human p46 and p48 can functionally replace multicopy *MSI1* in suppressing the heat shock sensitivity of yeast *ira1* and *RAS2*^{Val19} mutations (Qian et al., 1993; Qian and Lee, 1995), both of which result in elevated levels of cAMP. These four proteins form a highly conserved subgroup of the larger family of WD-repeat proteins (Figure 1A, here, and Neer et al., 1994). Thus, two of the subunits of human CAF-1 contain multiple WD-repeat motifs: CAF-1 p48 has seven repeats while CAF-1 p60 has from five to seven such repeats (Kaufman et al., 1995). It seems possible that the p46 and p48 proteins will assume a propeller structure similar to the β subunit of transducin, which also contains seven WD-repeat units (Neer and Smith, 1996, and references therein). In addition, the four p48-related proteins share a region of extensive amino acid sequence similarity near the amino terminus, a region that does not contain any WD repeat (Figure 1A).

p48 Is Associated with Histone H4 in the Absence of the Other Subunits of CAF-1

Both p46 and p48 are present in much larger amounts than the p150 and p60 subunits of CAF-1 in the S100 DNA replication extract that was used for chromatin assembly (Figure 2, lanes 1 and 2). So far, it has not been possible to immunodeplete either p46 or p48 from the S100 extract. As a result, it is not currently possible to address the issue of whether p48 is absolutely required for CAF-1 activity. Interestingly, however, a small proportion of the p48 protein was immunoprecipitated using affinity-purified antibodies against histone H4 but not with control antibodies (Figure 2, lanes 3–6). In contrast, although the p46 protein was present in large excess over p48 in the S100 extract (Figure 2, lane 2), no p46 protein was detectable in the anti-H4 immunoprecipitate (Figure 2, lanes 5 and 6, lower blot). Moreover, neither CAF-1 p150 nor p60 was detectable in the S100

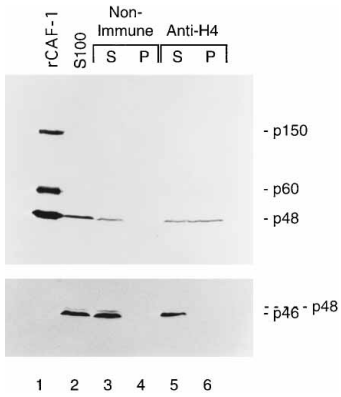

Figure 2. p48 Is Associated with Histone H4 in the Absence of CAF-1 p150 and p60 in Human Cell S100 Extract

Proteins from human 293 cell S100 extract and immunoprecipitates with anti-histone H4 antibody were detected by immunoblotting with antibodies against p150 (mAb p150-1), p60 (mAb p60-53), p48 (mAb 11G10, upper blot), p46 and p48 (mAb 15G12, lower blot). Lane 1: rCAF-1 used as a stoichiometry standard for the three subunits of CAF-1. Lane 2: S100 extract (1/20th of total). Lanes 3 and 4: immunoprecipitation from 90 μl of S100 extract with IgGs purified from nonimmune serum showing 1/20th of the supernatant (lane 3) and all of the pellet (lane 4). Lanes 5 and 6: immunoprecipitation from 90 μl of S100 extract with affinity-purified anti-H4 antibodies showing 1/20th of the supernatant (lane 5) and all of the pellet (lane 6). Overexposure of the lower blot does reveal the presence of p48 in lanes 5 and 6 (not shown).

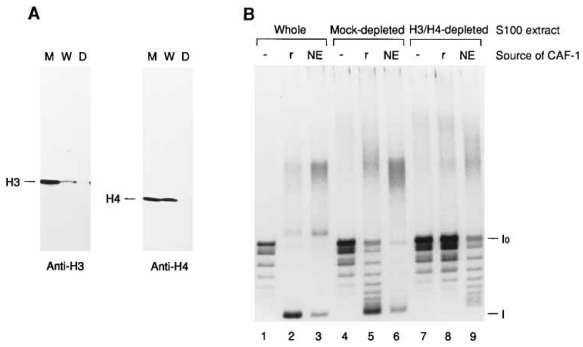

Figure 3. Nuclear Extracts from Human 293 Cells Contain an Activity That Promotes Chromatin Assembly in a Histone H3/H4-Depleted S100 Extract

(A) Immunodepletion with antibodies against histone H4 removes both histones H3 and H4 from the S100 extract. Immunoblots of whole (lane W) and immunodepleted (lane D) S100 extract probed with an antiserum against histone H3 and an antiserum against acetylated H4. Lane M of each panel contains total histones from human 293 cells as molecular weight markers.

(B) Supercoiling assays for chromatin assembly in the absence of CAF-1 (lane -) or presence of either recombinant CAF-1 (lane r) or human 293 cell nuclear extract (lane NE) performed using whole, mock-depleted, or histone H3/H4-depleted S100 extract. The positions of relaxed covalently closed DNA (form Io) and supercoiled DNA (form I) are indicated.

extract or in any of the immunoprecipitates (Figure 2), indicating that the p48 subunit of CAF-1 is bound to the histones in the absence of the two large subunits.

Identification and Purification of Chromatin Assembly Complex

In published work, we obtained evidence that some of the CAF-1 protein present in nuclear extracts of human 293 cells is physically associated with newly synthesized histones H3 and H4 (Kaufman et al., 1995). The H4 in this complex is recognized by antibodies specific for the acetylated N-terminal domain of H4 (Kaufman et al., 1995). To purify and characterize this CAF-1-histone complex, a functional assay was developed by immunodepleting histones from a cytosolic S100 extract of human 293 cells, which normally provides both the DNA replication factors and the newly synthesized histones required for chromatin assembly. As shown in Figure 3A, affinity-purified antibodies raised against a peptide derived from the amino terminus of H4 depleted both histones H3 and H4, in agreement with previous data showing that H3 and H4 are physically associated with each other in the S100 extract (Perry et al., 1993; Kaufman et al., 1995). The immunodepleted extract will be referred to as an H3/H4-depleted S100 extract. We then analyzed the ability of either recombinant CAF-1 (purified from Sf9 cells coinfected with baculoviruses expressing p150, p60, and p48, see Experimental Procedures) or a crude nuclear extract from human 293 cells to support chromatin assembly in three types of S100 extract. As illustrated in Figure 3B, both recombinant CAF-1 (rCAF-1) and nuclear extract can reconstitute chromatin assembly in both whole and mock-depleted S100 extracts. This simply reflects the fact that both rCAF-1 and human CAF-1 from the nuclear extract can recruit newly synthesized histones from the S100 extract and deposit them onto replicating DNA (Figure 3B, lanes 1–6). In contrast, when histones H3 and H4 have been immunodepleted from the S100 extract, only the crude nuclear extract from human cells can support chromatin assembly (Figure 3B, lanes 7–9).

As a first purification step, the crude nuclear extract was applied to a Q-Sepharose column at low ionic strength and the column was developed with a linear salt gradient. The fractions were analyzed for chromatin assembly activity in both the whole and H3/H4-depleted S100 extracts. When the Q-Sepharose fractions from the nuclear extract were analyzed in the whole S100 replication extract, two peaks of activity were detected (Figure 4A; flow-through and fractions 28–32). In contrast, only the Q-Sepharose flow-through fraction had chromatin assembly activity when the same fractions were analyzed in the H3/H4-depleted S100 extract (Figure 4B). Since chromatin assembly in the S100 extract requires CAF-1, these results argue that CAF-1 exists in at least two biochemically separable forms in the starting nuclear extract, only one of which is capable of supporting chromatin assembly in the H3/H4-depleted extract. The Q-Sepharose fractions were also analyzed by immunoblotting with antibodies against the three CAF-1 subunits and acetylated histone H4. The fractions that contain the two activities capable of complementing chromatin assembly in the whole S100 extract contain the three subunits of CAF-1 (Figure 4C). Only the Q-Sepharose flow-through fraction, however, contains acetylated histone H4 (Figure 4C), consistent with the fact that both CAF-1 and newly synthesized histones are required for chromatin assembly in the H3/H4-depleted

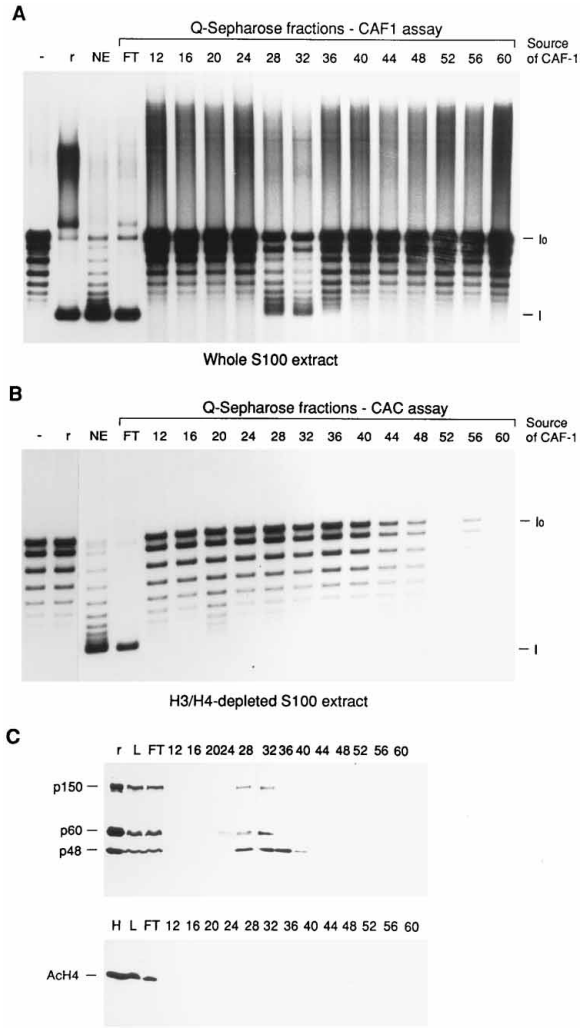

Figure 4. Separation of Two Biochemically Distinct Forms of CAF-1 from Nuclear Extracts of Human 293 Cells

(A) Supercoiling assays of Q-Sepharose fractions using whole S100 extract. Reactions without CAF-1 (lane -) and with recombinant CAF-1 (lane r) are also shown as controls.

(B) Supercoiling assay of Q-Sepharose fractions using a histone H3/H4-depleted S100 extract.

(C) Immunoblots of Q-Sepharose fractions probed with antibodies recognizing p150 (mAb p150-1), p60 (mAb p60-53), p48 (mAb11G10), and histone H4 acetylated at lysine 12. Lanes r and H: recombinant CAF-1 and histones used as molecular weight markers. Lane L: Q-Sepharose load. Lane FT: Q-Sepharose flow-through.

S100 extract. Finally, it is clear from Figure 4C that the most abundant form of the CAF-1 protein is the one present in the Q-Sepharose flow-through, as equal volumes of the Q-Sepharose load and flow-through fractions were analyzed by immunoblotting.

CAC Contains the Three Subunits of CAF-1 and Posttranslationally Modified Histones H3 and H4

The activity responsible for chromatin assembly in the H3/H4-depleted S100 extract was purified from the Q-Sepharose flow-through using an immunoaffinity procedure (see Experimental Procedures), followed by a final glycerol gradient sedimentation step. Since the glycerol gradient fractions were too dilute to be analyzed directly for chromatin assembly using the supercoiling assay, we used a more sensitive assay that detects the formation of micrococcal nuclease-resistant nucleosome cores onto newly synthesized DNA. This assay is based on the fact that the DNA in close contact with the histone octamer in the nucleosome core (~146 bp) is strongly protected against the exonuclease activity of micrococcal nuclease (Noll and Kornberg, 1977). The material loaded onto the final glycerol gradient was capable of promoting the formation of micrococcal nuclease-resistant nucleosome cores whereas rCAF-1 was completely inactive in this assay (Figure 5A). The activity applied to the gradient sedimented with an apparent molecular mass of ~150 kDa, which is similar to rCAF-1 but significantly larger than purified $H3_2.H4_2$ tetramers sedimented in parallel glycerol gradients (Figure 5B, open arrow). Active fractions were pooled, concentrated, and then assayed for chromatin assembly using the supercoiling assay described earlier. Although both rCAF-1 and purified CAC can support the formation of nucleosomal arrays in the whole S100 extract, only CAC can promote chromatin assembly in the H3/H4-depleted extract (Figure 5C).

The nucleosome assembly activity observed with the micrococcal nuclease digestion assay cosedimented with a set of five polypeptides that comigrated with CAF-1 p150, p60, p50, and histones H3 and H4 in SDS–polyacrylamide gels (Figures 5B and 6A). The identity of these polypeptides was confirmed by immunoblotting of glycerol gradient–purified CAC with antibodies recognizing the three subunits of CAF-1 and histones H3 and H4 (Figures 6B–6D). A monoclonal antibody that recognizes p48, but not p46 (mAb 11G10; Qian and Lee, 1995), clearly detected the 50 kDa subunit of CAC, arguing that the p48 polypeptide is a component of CAC. We have confirmed that this is the case by amino acid sequencing of the ~50 kDa polypeptide purified as a component of CAC. Three of the peptides sequenced (underlined with a dashed line in Figure 1A) are derived from the p48 polypeptide, and not p46, indicating that p48 is the small subunit of CAF-1 present in CAC. As p48 has been reported previously to bind to the retinoblastoma protein (Qian et al., 1993; Qian and Lee, 1995), we investigated whether Rb was associated with purified CAC. Immunoblots of purified CAC probed with affinity-purified polyclonal antibodies against Rb (which recognize both unphosphorylated and phosphorylated forms of Rb) did not detect any Rb protein in purified CAC (Figure 6E). The pRb-related proteins p107 and p300 were also undetectable (data not shown). Furthermore, because p48 exhibits extensive amino acid sequence similarity with yeast Hat2p, a component of a S. cerevisiae B-type histone H4 acetyltransferase (Parthun et al., 1996), we sought to determine whether purified CAC has histone acetyltransferase (HAT) activity. Immunoaffinity-purified CAC has no detectable HAT activity and weak HAT activities present in partially purified CAC preparations did not copurify with CAC (data not shown).

Newly synthesized H4 isolated shortly after its incorporation into chromosomes exhibits a very specific and

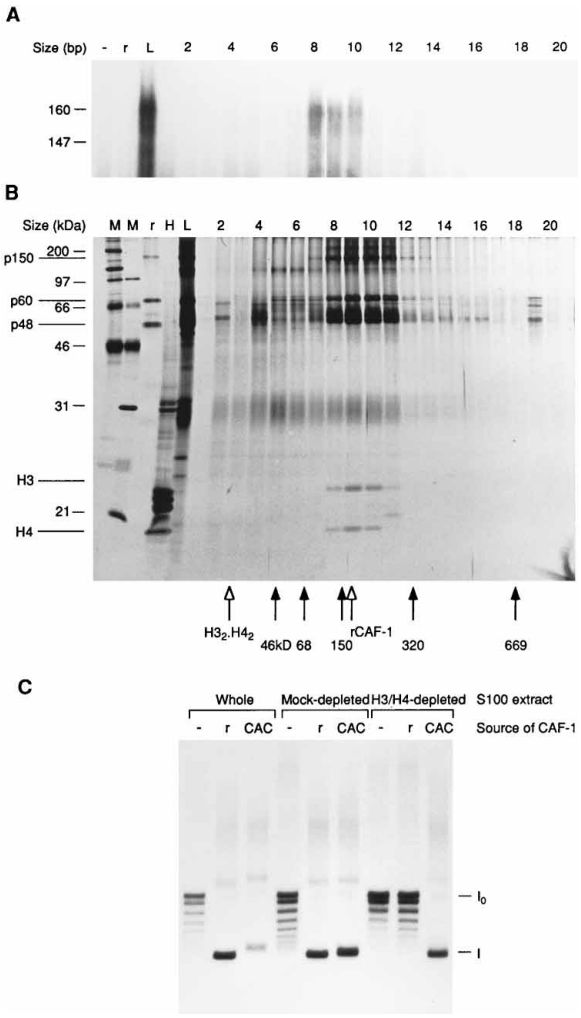

Figure 5. Glycerol Gradient Sedimentation of the Chromatin Assembly Complex (CAC)

(A) Micrococcal nuclease digestion assays of fractions across a glycerol gradient of CAF-1 immunoaffinity purified from a Q-Sepharose flow-through of 293 cell nuclear extract. Lane -: no factor added. Lane r: recombinant CAF-1. Lane L: glycerol gradient load.

(B) Fractions from the same glycerol gradient analyzed by electrophoresis through an SDS-18% polyacrylamide gel and silver staining. The positions of protein size markers, recombinant CAF-1 and purified core histone tetramers sedimented in parallel gradients are indicated at the bottom. Lane M: molecular weight markers. Lane r: recombinant CAF-1. Lane H: histone markers. Lane L: glycerol gradient load.

(C) Supercoiling assays in whole, mock-depleted or H3/H4-depleted S100 extract performed without CAF-1 (lane -) or with either recombinant CAF-1 (lane r) or purified CAC.

highly conserved pattern of acetylation in which lysines 5 and 12 are modified (Sobel et al., 1995). To find evidence that the histones in purified CAC are newly synthesized molecules, we analyzed their pattern of acetylation in acetic acid–urea gels, which resolve histones on the basis of both their size and net charge. Both H3 and H4 of CAC are heterogeneous mixtures of molecules with various degrees of posttranslational modification (Figures 7A and 7B). CAC H3 is a mixture of two isoforms that comigrate with the unacetylated (~60% of total CAC H3) and monoacetylated isoforms of H3 isolated from bulk chromatin. It is not possible to unambiguously attribute this modification of H3 to acetylation because either phosphorylation or acetylation would cause an upward shift in the electrophoretic mobility of H3 in acetic acid–urea gels (Barratt et al., 1994). We note, however, that antibodies against phosphoserine, phosphothreonine, or phosphotyrosine (Sigma), which recognize phosphorylation sites in a nonsequence-specific manner, do not detect phosphorylation of CAC H3 or H4 (Ruiz-Carrillo et al., 1975), whereas they readily detect phosphorylation of CAF-1 p150 and p60 in purified CAC (data not shown). Thus, it is likely that the posttranslational modification of CAC H3 seen in acetic acid–urea gels is attributable to acetylation.

CAC H4 is a mixture of four isoforms that migrate with unacetylated (~33% of total CAC H4 in Figure 7B), mono- (33%), di- (33%), and triacetylated H4 (undetectable in Figure 7B, but clearly detectable with acetylation-specific antibodies, Figures 7C and 7D). In addition, the overall level of acetylation of CAC H4 is markedly higher than that of H4 isolated from bulk 293 cell chromatin (which is predominantly un- and monoacetylated H4, Figure 7B). To determine which lysine residues are modified in CAC H4, we have probed immunoblots of CAC from acetic acid–urea gels with site-specific antibodies that recognize H4 molecules modified at each of the four known sites of H4 acetylation (Turner and Fellows, 1989). Acetylated isoforms of CAC H4 can be detected with three of the acetylation site-specific antisera, namely those recognizing H4 acetylated at lysine 5, 8, or 12, but not with that recognizing H4 acetylated at lysine 16 (Figure 7). Consistent with the presence of up to three sites of acetylation in CAC H4, the antisera specific for H4 acetylated at lysines 5 or 8 both recognized an isoform of H4 that comigrates with the triacetylated histone H4 marker (Figures 7C and 7D). Hence, with the exception of lysine 8 acetylation, the pattern of CAC H4 acetylation is consistent with that reported for newly synthesized H4 isolated shortly after its incorporation into chromosomes (Sobel et al., 1995).

The p48 Subunit of CAF-1 Exists in at Least Two Biochemically Distinct Forms in Human Cell Nuclear Extracts

During the course of purification of the chromatin assembly complex, we monitored the abundance of the three CAF-1 subunits before and after immunoaffinity purification. Surprisingly, although a monoclonal antibody against p150 routinely immunodepleted most of CAF-1 p150 and p60, this antibody depleted very little CAF-1 p48 (data not shown). This suggested that most of the p48 polypeptide present in the crude nuclear fraction used as starting material for purification of CAC was not actually associated with CAC. To demonstrate this, the starting material for the purification was sedimented through a glycerol gradient and the fractions analyzed by immunoblotting with monoclonal antibodies against the three subunits of CAF-1. As anticipated, most of the p48 polypeptide did not cosediment with CAC (Figure 8). Interestingly, the bulk of the p48 protein sedimented with an apparent molecular weight of ~320 kDa (fraction 13 in Figure 8), which is significantly larger

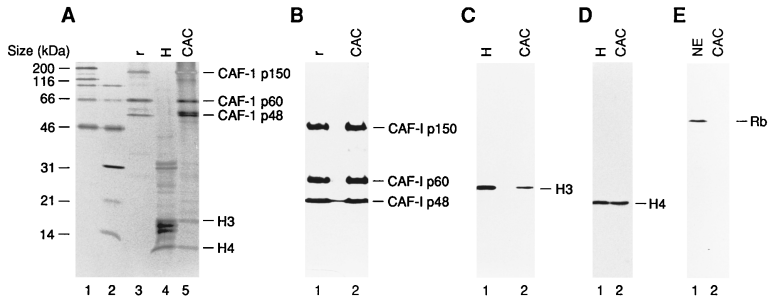

Figure 6. Purified Chromatin Assembly Complex contains CAF-1 p150, p60, p48, and Core Histones H3 and H4

(A) Coomassie-stained SDS–18% polyacrylamide gel of purified CAC. Lanes 1 and 2: molecular weight markers. Lane 3: recombinant CAF-1. Lane 4: histone markers. Lane 5: CAC.

(B) Immunoblot probed with antibodies recognizing CAF-1 p150 (mAb p150–1), p60 (mAb p60–53), and p48 (mAb11G10; Qian and Lee, 1995). Lane 1: recombinant CAF-1. Lane 2: CAC.

(C) Immunoblot probed with an antiserum against histone H3. Lane 1: histone markers. Lane 2: CAC.

(D) Immunoblot probed with an antiserum against histone H4. Lane 1: histone markers. Lane 2: CAC.

(E) Immunoblot probed with affinity-purified anti-Rb polyclonal antibodies. Lane 1: nuclear extract from 293 cells. Lane 2: CAC.

than CAC (~150 kDa, fraction 9 in Figure 8). Thus, most of the p48 protein in this crude nuclear fraction of human cells is present in a complex that is biochemically distinct from CAC. As expected, this higher molecular weight p48-containing complex is inactive in our cell-free system for de novo nucleosome assembly (data not shown), because it lacks the essential CAF-1 p150 and p60 subunits (Kaufman et al., 1995).

Discussion

p48 Is the Small Subunit of CAF-1

The description of p48 as the small subunit of CAF-1 completes the identification of the three previously described CAF-1 polypeptides (Smith and Stillman, 1989). So far, unlike for the p150 and p60 subunits (Kaufman et al., 1995), the available antibodies do not immunodeplete p48 from the cytosolic extract used for in vitro chromatin assembly, so that we cannot yet conclude that this polypeptide is required for CAF-1 activity. However, there is no doubt that p48 is an intrinsic component of CAC since the p48 polypeptide is coimmunoprecipitated by antibodies against the other subunits of CAF-1 or anti-H4 antibodies. This suggests that p48 may function in stoichiometric amounts with the other two subunits of CAF-1 to promote chromatin assembly. The homolog of the CAF-1 p48 subunit from Drosophila (dCAF-1 p55) was recently isolated as a subunit Drosophila CAF-1 (Tyler et al., 1996).

The association of p48 with histone H4 in the absence of the other subunits of CAF-1 may represent an early event in the nucleosome assembly pathway. We have previously demonstrated that addition of CAF-1 p150 to an S100 extract allows coimmunoprecipitation of a complex of newly synthesized H3 and H4 with anti-p150 antibodies (Kaufman et al., 1995). Therefore, in vivo, p48 may first bind to newly synthesized histones H3 and H4 and facilitate the assembly of the other subunits of CAF-1 onto the core histones to generate the chromatin assembly complex. Detailed biochemical studies are underway to determine whether the p48 subunit is critical for CAF-1 to bind to the core histones.

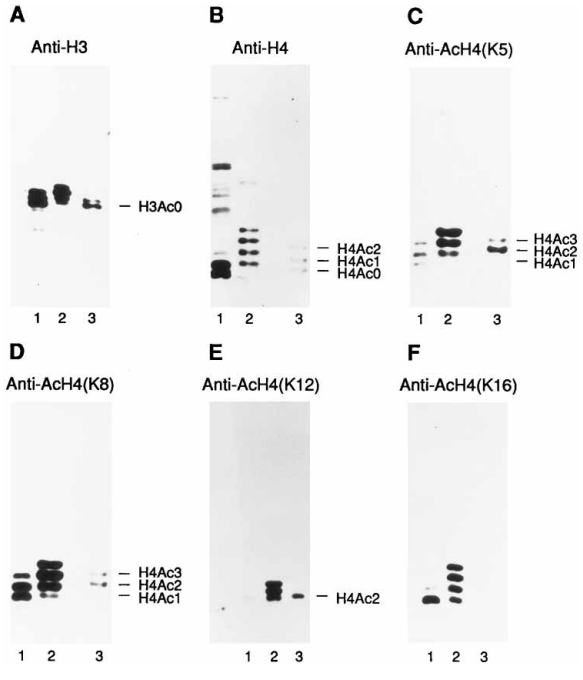

Figure 7. CAC Histone H4 Is a Heterogeneous Mixture of Molecules with Zero, One, Two, and Three Acetyl Groups per Molecule That Are Modified at Lysines 5, 8, and 12

Immunoblots of CAC polypeptides separated by acetic acid–urea gel electrophoresis and probed with antibodies specific for histone H3 (A), H4 (B), and H4 acetylated at lysine 5 (C), 8 (D), 12 (E), or 16 (F). The position of specific isoforms of H3 and H4 is indicated along the right-hand side of each panel. Lane 1: total histones from 293 cell chromatin Lane 2: hyperacetylated histones H3 and H4 isolated from chromatin of butyrate-treated 293 cells. Lane 3: CAC.

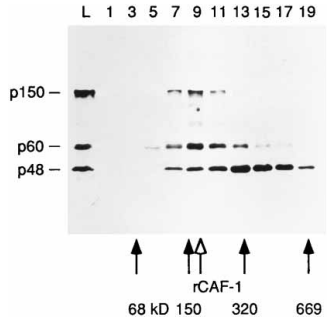

Figure 8. Biochemically Distinct Complexes Containing p48 in Human 293 Cells

Glycerol gradient sedimentation of the crude nuclear extract fraction from which CAC was purified. Fractions from the glycerol gradient were immunoblotted and probed with antibodies recognizing CAF-1 p150 (mAb p150-1), p60 (mAb p60-53), and p48 (mAb11G10). Lane L is the material loaded onto the gradient. The position of protein size markers and recombinant CAF-1 that were sedimented in parallel gradients is indicated at the bottom.

The finding that none of the large amount of p46 (which is 89% identical to p48) present in the S100 extract is bound to H4, is intriguing in the light of experiments with recombinant p46 and p48 which show that both proteins bind directly and with high affinity to any of the four types of core histones (A. Verreault, unpublished data). It is possible that the p46 protein in the S100 extract may be posttranslationally modified or bound to some other protein, and thereby prevented from binding to histone H4.

Several lines of evidence argue that p48 may play other cellular functions aside from its role in chromatin assembly. First, p48 is tightly associated with the HD1 protein, the catalytic subunit of a human histone deacetylase (Taunton et al., 1996). HD1 is related to the S. cerevisiae Rpd3p transcriptional regulator, suggesting that p48 may have roles in the regulation of gene expression and/or the deacetylation of newly synthesized histones that occurs shortly after their incorporation into chromosomes. Second, as shown herein, a large amount of p48 also exists in a large protein complex of unknown biochemical composition. Third, the amino acid sequence similarity between human p46 and p48 and S. cerevisiae Hat2p and Msi1p is striking. Hat2p is the histone binding subunit of a yeast B-type histone H4 acetyltransferase and its association with the catalytic subunit (encoded by the *HAT1* gene; Kleff et al., 1995) strongly enhances the specific activity of the enzyme (Parthun et al., 1996). It therefore seems likely that p46, p48, Hat2p, and Msi1p form an evolutionarily conserved subfamily of WD-repeat proteins that function in chromatin assembly, histone acetylation, deacetylation, and possibly other cellular processes. The fact that members of this family of proteins participate in various aspects of histone metabolism suggests that these proteins may function generally as histone chaperones by attracting proteins such as CAF-1, histone acetyltransferases, and deacetylases to the histones.

Although both p46 and p48 were originally isolated from human cell extracts as proteins that associate with an immobilized fragment of the retinoblastoma protein (Qian et al., 1993; Qian and Lee, 1995), we have not been able to find any evidence that Rb associates with CAF-1. In addition, Rb is clearly not associated in stoichiometric amounts with the purified histone deacetylase HD1.p48 complex (Taunton et al., 1996). Thus, the in vivo significance of Rb binding to p46 and p48 remains to be determined.

Chromatin Assembly Complex

Most of the CAF-1 protein in human 293 cell nuclei is part of CAC, which contains the three CAF-1 subunits (p150, p60, and p48) and posttranslationally modified histones H3 and H4. CAC H4 is acetylated at specific lysine residues in a manner consistent with, yet slightly distinct from, the known pattern of acetylation of newly synthesized histone H4 isolated from nascent chromosomes (Sobel et al., 1995). We find that lysine 8, in addition to lysines 5 and 12, of CAC H4 is acetylated. The reason for the difference between our data and those of Allis and coworkers is unknown. It may be due to the transient and dynamic nature of acetylation of newly sythesized histones in proliferating cells (Jackson et al., 1976). For instance, it is possible that the histones bound to CAF-1 represent a small, differently modified subset of the total newly synthesized H3 and H4, perhaps because the histones associated with CAF-1 are not susceptible to the deacetylase enzyme that acts shortly after deposition of the histones into nascent chromatin. Interestingly, there is considerable amino acid sequence similarity between the lysine 5, 8, and 12 acetylation sites ($GRGK_5GGK$, $KGGK_8GLG$, $GLGK_{12}GGA$) that are modified in CAC H4. In particular, the tripeptide GKG, which contains the acetylatable lysine, is present in the K5, K8, and K12 sites but is absent from the K16 site ($GGAK_{16}RHR$), suggesting that these sites may be the target of a common human B-type H4 acetyltransferase enzyme. Moreover, the three sites modified in CAC are related to the target site for the yeast B-type H4 acetyltransferase Hat1p (Parthun et al., 1996). It is plausible that the human counterpart of yeast Hat1p may prove capable of acetylating the three sites modified in CAC H4.

CAC H3 also displays a modification pattern that differs slightly from results published previously. In human cells, Allis and coworkers found no acetylation of newly synthesized HeLa cell histone H3 using their amino acid sequence methodology, although acetylation of up to two sites was found in Drosophila and Tetrahymena (Sobel et al., 1995). In contrast, our data show that ~40% of the CAC H3 molecules are modified, but we cannot unambiguously attribute the modification to phosphorylation or acetylation. Phosphorylation of H3 has been previously reported in a cell-free system for replication-independent chromatin assembly derived from HeLa cells (Banerjee et al., 1991).

Histone Acetylation and Chromatin Assembly

Although the sites of acetylation of newly synthesized H4 are highly conserved in widely divergent organisms (Sobel et al., 1995), the N-termini of H3 and H4, which contain all the sites of acetylation, are not individually essential in S. cerevisiae (Morgan et al., 1991; Ling et al., 1996). However, a deletion of the N-termini of both H3 and H4 in the same yeast strain is not viable (Morgan et al., 1991; Ling et al., 1996), and cells lacking both the H3 and H4 amino termini may have a defect in chromatin assembly (Ling et al., 1996). These data argue that the N-terminal domains of H3 and H4 play an essential yet redundant function in chromatin assembly, but it is not yet known if this function involves acetylation of these domains.

In vivo, there is a close temporal relationship between histone synthesis, acetylation, and deposition into chromatin (Jackson et al., 1976; Worcel et al., 1978; Lin et al., 1989; Sobel et al., 1995). CAF-1 is associated with newly synthesized and acetylated histones, suggesting that acetylation may play a role in targeting newly synthesized histones to sites of DNA replication via transport by CAF-1. It will be important to discern in future work whether the acetylation state of the amino-terminal domains of H3 and H4 is important for nucleosome assembly by CAF-1 per se, rather than for cellular localization or another in vivo function.

At least in human cells, it is possible that acetylation of newly synthesized histones may also be required to

regulate chromosome maturation because treatment of tissue culture cells with inhibitors of histone deacetylase blocks maturation of nascent chromatin, resulting in misincorporation of histone H1 and chromatin that remains sensitive to DNase I (Perry and Annunziato, 1991, and references therein). The association of p48 with both CAF-1 and the catalytic subunit of histone deacetylase (Taunton et al., 1996) suggests that deacetylation of nascent chromatin, a prerequisite for chromosome maturation, may be carried out by HD1. Finally, it is also possible that the transient acetylation of newly synthesized histones may play additional roles in processes unrelated to histone deposition. For instance, it has been proposed that deposition-related histone acetylation may provide a critical window of opportunity during which nonhistone DNA-binding proteins (e.g. transcription factors) may gain access to their binding sites in nascent, yet immature chromatin (Lee et al., 1993).

The data presented here lead us to postulate that CAC is an activated form of the CAF-1 protein in human cells and, therefore, an important mediator of de novo nucleosome assembly during S phase of the cell cycle. This does not, however, rule out that additional factors may be required for de novo nucleosome assembly or that other pathways may exist.

Experimental Procedures

Cloning of CAF-1 p48 Subunit

Human CAF-1 was purified from 293 cell nuclei and prepared for amino acid sequence analysis by "in-gel" digestion with lysylendopeptidase as described by Kaufman et al., 1995. Four peptide sequences were obtained from the p50 protein (Figure 1). Degenerate 17 nt primers were designed based on the N- and C-terminal six amino acids of the longest peptide obtained (21 residues; Figure 1). RT–PCR reactions using these primers and human 293 cell RNA produced a product of the expected (47 bp) size. This product was cloned and several of the isolates contained internal sequences encoding the amino acids predicted from the peptide sequence data. Nondegenerate primers including the internal bases of the 47 bp PCR product were then used in conjunction with primers based on the next largest peptide to amplify a 1.1 kb PCR product. DNA sequence analysis showed that this PCR product encoded amino acids predicted from the peptide sequence data that were not included in the amplification oligonucleotides. The 1.1 kb fragment was used to probe a HeLa λZAPII library (Stratagene). Numerous positives were identified and cloned, and in vivo excision of insert-containing clones was performed according to the manufacturer's instructions. A cDNA of 1.8 kb (pPK10) was sequenced on both strands with Sequenase II (USB). This gene is now known to encode the p46 protein isolated as an Rb-binding protein (Qian and Lee, 1995). The p46 protein is highly homologous to p48 (Figure 1), and two of the peptides sequenced were present in the p46 sequence. One peptide near the N-terminus was similar, but not identical to the p46 sequence; another had a single amino acid difference. Subsequent publication of the sequence of the p48 cDNA (Qian et al., 1993) showed that all the correct peptide sequences were present in p48.

Other DNA Manipulations

A p48-encoding cDNA in plasmid pGEM-RbAp48 (Qian et al., 1993) was subcloned as an EcoRI fragment into pBSKS+ (Stratagene) with the 5′ end closer to the SacI site of the polylinker (pPK32). DNA between the SmaI site in the polylinker and the NcoI site at the start codon was deleted, forming pPK35. A NotI–EcoRI fragment of pPK35 carrying the truncated cDNA was cloned into a baculovirus vector carrying the p10 promoter, forming pPK37. A recombinant baculovirus was generated by cotransfection of Sf9 cells with pPK37 DNA and BaculoGold linear viral DNA according to the manufacturer's instructions (Pharmingen).

Purification of CAC from Human 293 Cell Nuclear Extract

All purification steps were performed at 4°C, and buffers contained 10 mM sodium butyrate (pH 7.5) as a histone deacetylase inhibitor and the following protease inhibitors: 1 mM benzamidine, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. Nuclei derived from a 64-liter suspension culture of 293 cells were prepared and extracted with 0.4 M NaCl as described by Smith and Stillman (1989). An ammonium sulfate precipitate (25%–60% saturation) of this nuclear extract served as starting material for the purification. The ammonium sulfate pellet was resuspended in 40 ml of buffer A (25 mM Tris–HCl [pH 8.0], 10% glycerol, 0.01% NP-40, 1 mM EDTA, 1 mM DTT) and dialyzed against buffer A.

This crude nuclear extract was applied to a 60 ml Q-Sepharose column equilibrated in buffer A, washed with 300 ml of buffer A and developed with a 300 ml salt gradient from buffer A0 to buffer A800 (where the number indicates the millimolar concentration of NaCl added to buffer A). During this chromatographic step, CAC activity eluted in the 0 M NaCl flow-through. The Q-Sepharose flow-through was adjusted to 0.35 M NaCl and loaded onto a 2 ml column of monoclonal antibody against T antigen (pAb 419) that had been cross-linked to protein A–Sepharose with dimethylpimelimidate (Harlow and Lane, 1988). The flow-through from this step was incubated overnight with 1.5 ml of protein G–Sepharose to which a monoclonal antibody against CAF-1 p150 (mAb p150-1, partially purified from 1.5 ml of ascites fluid by ammonium sulfate fractionation) had been cross-linked covalently using dimethylpimelimidate (Harlow and Lane, 1988). The affinity column was then washed with 150 ml of buffer A350 and eluted with 7.5 ml of buffer A + 5 M LiCl. The affinity column eluate was dialyzed against buffer A25 and concentrated 20-fold by dehydration with sucrose.

For sedimentation analysis, CAC was loaded onto a 5 ml, 15%–35% linear glycerol gradient in buffer A150. Recombinant CAF-1, core histone tetramers, and protein size markers were run in parallel gradients to compare their respective rates of sedimentation with that of CAC. When crude nuclear extract fractions were analyzed by sedimentation, the gradients were prepared in buffer A400 plus 100 μg/ml ethidium bromide to minimize the association of protein with nucleic acids (Lai and Herr, 1992). Glycerol gradients were centrifuged in a Beckman SW55Ti rotor at 50,000 rpm for 15 hr. The gradients were fractionated into twenty 250 μl fractions and the polypeptide composition of the fractions assayed by electrophoresis in SDS–18% polyacrylamide gels (see below) and silver staining. The CAC gradient fractions were also assayed for their chromatin assembly activity in the H3/H4-depleted S100 extract using a micrococcal nuclease digestion assay (see below). CAC polypeptides were sequenced as described above for purified CAF-1.

Purification of Recombinant Human CAF-1 Expressed in Sf9 Cells

Recombinant human CAF-1 (rCAF-1) was expressed by coinfection of Sf9 cells with recombinant baculoviruses expressing each of the CAF-1 subunits (Kaufman et al., 1995). All the purification steps were performed at 4°C; all buffers contained the cocktail of protease inhibitors used for the purification of CAC (see above). A nuclear extract of infected Sf9 cells was prepared as described in Kaufman et al. (1995). rCAF-1 was then isolated by immunoaffinity purification using mAb p150-1 exactly as described above for CAC. rCAF-1 was dialyzed against buffer B (10 mM potassium phosphate [pH 7.5], 10% glycerol, 0.01% NP-40, 1 mM EDTA, 1 mM DTT) + 25 mM NaCl and applied to a 0.1 ml hydroxyapatite column equilibrated in buffer B + 25 mM NaCl. The column was washed with 4 ml of buffer B + 0.4 M NaCl, then with 4 ml of buffer B + 240 mM potassium phosphate pH 7.5, and finally eluted with buffer B + 790 mM potassium phosphate pH 7.5. rCAF-1 was dialyzed extensively against buffer A25.

Purification of Core Histones from Butyrate-Treated Human 293 Cells

To induce histone hyperacetylation, 10 mM sodium butyrate (pH 7.5) was added to a suspension culture of human 293 cells (~1 ×

10^6 cells/ml), grown as described by Stillman and Gluzman (1985), and the cells were harvested 16–20 hr later. All the purification steps were performed at 4°C, and all buffers contained the cocktail of protease inhibitors used for the purification of CAC (see above), 10 mM sodium butyrate (pH 7.5) and 1 mM DTT. Nuclei from a 16-liter culture of 293 cells were prepared as described previously (Smith and Stillman, 1989) and lysed in 1 mM Na_2EDTA (pH 7.5), 1 mM Na_2EGTA (pH 7.5) for 15 min. Chromatin was sheared by sonication and insoluble debris was removed by centrifugation (20 min, 12,000 × g). H2A.H2B dimers and $H3_2.H4_2$ tetramers were then purified by binding soluble chromatin fragments to hydroxyapatite and stepwise histone elution with increasing salt concentrations (Simon and Felsenfeld, 1979).

Supercoiling Assay for Chromatin Assembly

The S100 extract for SV40 DNA replication was prepared from suspension cultures of human 293 cells as described previously (Stillman and Gluzman 1985). SV40 T antigen was expressed by infection of Sf9 insect cells with recombinant baculovirus 941T (Lanford, 1988) and purified by immunoaffinity chromatography using monoclonal antibody pAb419 according to Simanis and Lane (1985).

The assays were performed as described previously (Stillman, 1986; Smith and Stillman, 1991) with the following modification: DNA replication reactions were performed for 45 min at 37°C prior to the addition of 3.2 μg/ml H2A.H2B dimers (purified from 293 cells that had not been treated with sodium butyrate). Incubation at 37°C was then resumed for 45 min prior to DNA extraction and analysis of the products by agarose gel electrophoresis and autoradiography.

Micrococcal Nuclease Digestion Assay for Chromatin Assembly

For these assays, DNA replication reactions were performed exactly as described for supercoiling assays above. After the second 45 min incubation, $CaCl_2$ and micrococcal nuclease were added to the reaction to 3 mM and 16 U/ml (Worthington), respectively. Micrococcal nuclease digestion was performed at 16°C for 1 hr and DNA extracted as described previously (Smith and Stillman, 1991). The products of micrococcal nuclease digestion were analyzed by electrophoresis in nondenaturing 5% polyacrylamide gels and autoradiography (Sambrook et al., 1989).

Immunological Procedures

An antiserum against the amino terminus of histone H4 acetylated at lysine 12 was raised by coupling peptide CSH463 (H-CGKGL GacKGGAKY-NH_2, where acK is ε-N-acetyl lysine) to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce) according to manufacturer's instructions. Rabbits were first immunized with 500 μg of KLH.CSH463 conjugate, followed by additional injections of 200 μg every other week. A matrix to affinity-purify antibodies that bind to the CSH463 peptide was prepared by cross-linking the peptide to Sulfo Link resin (Pierce). Anti-CSH463 antibodies were affinity purified as described in Harlow and Lane (1988). To prepare a resin for immunodepletion of histones from the S100 extract, 800 μg of affinity-purified antibodies were incubated with 500 μl of protein A–Sepharose in phosphate-buffered saline. This resin (20 μl) was incubated overnight with 100 μl of S100 extract and the supernatant assayed for histone immunodepletion by immunoblotting. The same resin (10 μl) was also used for coimmunoprecipitation of H4 and p48 from 90 μl S100 extract diluted with buffer A400 + 100 μg/ml ethidium bromide.

Histones were immunoblotted from SDS–18% polyacrylamide gels (Thomas and Kornberg, 1978) onto nitrocellulose in 10 mM CAPS (NaOH; pH 11), 20% methanol for 2 hr at 70 V. Acetic acid (0.9 M)–urea (2.5 M)–15% polyacrylamide gels were cast and run as described by Davie (1982), except that they were subjected to electrophoresis for much longer (10 hr at 200 V for 13 cm long, 0.7 mm thick gels) in order to resolve all the acetylated forms of both H3 and H4. Prior to electrophoretic transfer, acetic acid–urea polyacrylamide gels were processed according to the procedure described in Delcuve and Davie (1992) and histones transferred to nitrocellulose as above.

Although CAF-1 p48 and p60 (which migrates with an apparent molecular weight of ~66 kDa) can be immunoblotted efficiently from SDS–18% polyacrylamide gels, the CAF-1 p150 subunit cannot. To detect the three subunits of CAF-1 simultaneously by immunoblotting, the proteins were separated by electrophoresis through SDS–10% polyacrylamide gels (Laemmli, 1970). Antisera against core histones H3 and H4 (i.e. antisera that bind to both unacetylated and acetylated forms of H3 and H4) were provided by Dr. E. P. Bers (Univ. St Petersburg, Russia). Antisera specific for histone H4 acetylated at lysines 5, 8, 12, or 16 were provided by Dr. B. Turner (Turner and Fellows, 1989). A monoclonal antibody that binds specifically to p48 and not to p46 (mAb11G10) and an antibody that binds equally well to p46 and p48 (mAb15G12) were provided by Dr. E. Lee (Qian and Lee, 1995). Affinity-purified anti-Rb polyclonal antibodies were obtained from a commercial supplier (Santa Cruz) or the Cold Spring Harbor Laboratory monoclonal antibody facility.

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